CHAPTER 3
RESULTS AND DISCUSSION

3.1 BIO-POLYMERIC SCAFFOLDING APPROACH ON METALLIC Ti

3.1.1 INTRODUCTION

Titanium (Ti) and its alloys are widely used in biomedical devices as bone tissue replacements due to their exceptional mechanical and biological properties compared to other metallic biomaterials [201]. However, the problem of implant loosening followed by revision surgery is still a great source of concern in the biomedical field [202]. Numerous efforts are being taken in this field to improve the life expectancy of orthopaedic implants. Since the surface property of implants play a major role in determining the osseointegration, great efforts are being taken to improve biocompatibility of current implant devices by modifying Ti surfaces using different novel mechanical and chemical treatments. This include surface roughening by sand blasting [7], immobilizing TiO$_2$ nanofibers on to Ti surface by electrospinning [8], giving thin film coating of hydroxyapatite by plasma spraying, etc [203]. All of these approaches have shown varying degrees of success. Despite its significant success as a biomaterial, scientists are actively interested in investigating the surface property of Ti implants to address difficult clinical scenarios. Apart from the traditional surface modification techniques, nowadays significant research effort is aimed at the biochemical modifications of titanium surfaces. Numerous previous articles have pointed out the importance of biochemical modifications in regulating differentiation and remodelling of cells and tissue. Biochemical surface modification utilizes critical organic components of bone to affect specific tissue response [43]. This approach in particular involves the immobilization of biological components such as ECM molecules.
Collagen I, RGD peptides, fibronectin), Growth factors and peptides onto Ti substrates with the aid of crosslinking agents. One major factor responsible for enhancing the functions of cells onto different substrates introduced is the 3D organization and spatial distribution of these molecules. Though this molecules coated on Ti improved the cell function, the 3D organization of these materials is important in determining the desirable cell material interactions.

Here, we are proposing an alternative approach in which 3D biopolymeric scaffolding is generated on metallic Ti by a process of chemical cross linking with dopamine followed by lyophilization. The resulting 3D scaffold is microporous and nanofibrous in nature such that it facilitates the penetration of cells (hMSCs) into the scaffolds. Natural biopolymers such as fibrin and alginate are selected for fabricating the scaffolding on Ti due to their excellent biocompatibility properties [204, 205]. This approach has a further advantage that Ti can be pre-integrated with a surface bone layer prior to implantation. We envision that this method could be a better solution for improving the osteointegration of orthopaedic implants.

3.1.2 RESEARCH QUESTION AND HYPOTHESIS

**Question:** How can an adherent bio-polymeric scaffold of fibrin/alginate be immobilized on to metallic Ti?

**Hypothesis:** By adopting a biochemical modification technique wherein fibrin and alginate are immobilized on to Ti implants by chemical crosslinking with dopamine followed by lyophilisation, a biomimetic surface scaffolding can be developed on metallic titanium.

3.1.3 RESULTS AND DISCUSSION

**3.1.3.1 Surface Characteristics of the Fabricated Scaffolds on Ti**

A novel method for fabricating a microporous nanofibrous scaffold structure on metallic Ti was devised using biodegradable polymers such as fibrin and alginate in combination with dopamine as a crosslinker. Morphology of the Ti surfaces after the treatment procedures is shown in Figure 3.1.1. When fibrin
alone was used for the coating, it formed a disintegrated patchy network, which is clearly evident from the SEM image Figure 3.1.1 (b). On the contrary, when alginate was blended with fibrin, the composite coated plates exhibited a microporous nanofibrous network as shown in Figure 3.1.1(c). This scaffold architecture contributed by fibrin and alginate on metallic Ti showed a close resemblance to the extracellular matrix of normal bone as shown in Figure 3.1.1(d).

Fibrin together with alginate formed an interpenetrating network (IPN) of the two polymers, where one polymer is crosslinked in the presence of the other, either simultaneously or sequentially. Thrombin (resuspended in CaCl₂) treatment initiated the polymerization of fibrinogen into fibrin and concomitantly alginate also got crosslinked by calcium ions. IPNs can be advantageous because desirable properties of each individual polymer can be utilized in a single system [206]. Fibrin forms a relatively bioactive proteinaceous component with low mechanical strength, whereas alginate is a bioinert hydrogel with good mechanical strength [126, 135]. Reports say that there may or may not be any chemical bonds between the two networks. However, in the case of fibrin and alginate a possible electrostatic interaction can take place between the positively charged amino group of fibrin and negatively charged carboxylate group of alginate [207]. Thus, combination of these two natural polymers resulted in a completely entangled network, which immediately formed a hydrogel in the presence of thrombin and calcium. The lyophilization step used further to these chemical modifications helped in the generation of a 3D porous texture on Ti. The 3D nature of the formed scaffolding was further confirmed by a thickness analysis using surface profilometry. The average thickness of the Ti plates at each processing step i.e., after hydrothermal, dopamine and fibrin/alginate treatments were analyzed to be 300 ± 10 nm, 350 ± 20 nm and 109.44 ± 10.97 μm respectively. From the thickness analysis, it is clearly evident that fibrin/alginate treatment imparted a much thicker, mechanically adherent coating which will be demonstrated further.
Chapter 3  Results and Discussion

Figure 3.1.1: Scanning electron micrographic images of the scaffolding on Ti; (a) control polished Ti (b) shows the surface scaffolding on Ti with fibrin alone and (c) shows the surface scaffolding fabricated on Ti with fibrin and alginate. The fabricated fibrin/alginate scaffold in architecture is mimicking the extra cellular matrix of native bone (picture courtesy: International osteoporotic foundation) which is shown in (d). Higher magnification SEM micrographs of fibrin/alginate scaffold are shown on the right side (e and f).

Various studies have shown that, for osteointegration or bone bonding, the implant material should possess controlled pore size and interconnections, and nanostructured walls that can offer a favourable cell-material interaction [208]. The scaffolding which was fabricated on the Ti plates in the present work generated a microporous nanofibrous interpenetrating network with the nanofibrillary networks of fibrin and alginate being evident on the scaffold. It is reported that pore size in the range of 75-250 μm is suitable for bone growth [209]. A qualitative analysis of pore size on the scaffolding using SEM revealed values ranging from 50-100 μm. These values clearly correlate with that reported for bone trabeculae and osteons and would favour bone cell ingrowth, vascularization, as well as transport of metabolic products.

3.1.3.2 Topographic Studies using AFM

Figure 3.1.2 shows the representative AFM images of bare, hydrothermally treated, dopamine treated and fibrin/alginate deposited Ti plates, with their relative surface roughness profiles. It is clearly evident from the images that the bare Ti surface is relatively flat and uniform (Figure 3.1.2A). The average
surface roughness was found to be ~250 nm, whereas hydrothermally treated plate showed a rough surface morphology (~ 500 nm) (Figure 3.1.2B). This can be attributed to the nanoporous TiO₂ deposition on Ti facilitated by the hydrothermal processing. The surface roughness was found to be reduced upon passivation using dopamine (Figure 3.1.2C). However, the dopamine treatment of Ti plate resulted in the formation of a uniform, homogeneous and highly co-ordinated porous network, which is expected to improve its further biopolymer scaffolding. After fibrin/alginate deposition, the surface roughness increased considerably (~600 nm) indicating the successful adhesion of the bio-polymeric layer on Ti surface (Figure 3.1.2D).

![AFM images and roughness measurement](image)

Figure 3.1.2: AFM images and roughness measurement of A) control Ti plate; B) Ti plate after hydrothermal treatment; C) Ti plate after dopamine treatment and D) fibrin/alginate scaffolding.
Surface roughness is an important parameter that determines cell behaviour on the material both in vitro and in vivo [210]. Numerous reports have shown the changes in phenotypic expression of cells grown on surfaces with different surface roughness. It has been reported that rat osteoblasts when cultured on a rough surface (0.8 µm) showed higher proliferation and ALP activity compared to smooth surfaces [211]. It has been recommended that implants with a moderate surface roughness of 1-2 µm, can result in early fixation with bone cells and a long term mechanical stability of the implant [212]. Herein, our investigations on the changes in surface topography of the functionalized substrates by AFM analysis revealed an enhancement in surface roughness after dopamine treatment and its further interaction with fibrin/alginate, which in turn would promote bioactivity and cell growth.

### 3.1.3.3 XPS

XPS is a surface analysis technique which can provide both qualitative as well as quantitative information about different elemental compositions of the surface at different steps of surface functionalization. The wide scan spectra of control polished Ti, hydrothermally treated Ti, dopamine treated Ti and modified Ti and their corresponding surface elemental composition are shown in Figure 3.1.3 and Table 3.1.1 respectively. In the wide scan spectra of control Ti and hydrothermally treated Ti, three peaks were prominently seen. The predominant peaks were that of C 1s, Ti 2p and O 1s with respective binding energies (BE) of 285.0 eV for carbon, 458.0 and 463.6 eV for titanium and 531.0 eV for oxygen. Titanium and oxygen present in the CTi spectra might have evolved from the native TiO₂ layer present on the Ti plates and carbon due to unavoidable hydrocarbon contamination, which was used as an internal reference at 284.6 eV for peak position calibration [213]. After hydrothermal treatment, considerable increase in the oxygen intensity was noticed (refer Table 3.1.1), probably due to the deposition of TiO₂ layer. A small amount of nitrogen was also present in the wide scan spectra of control Ti and hydrothermally modified Ti which might be due to adventitious contamination. In contrast, after dopamine and fibrin/alginate treatment, a reduction in the Ti 2p signals were noticed with a sharp enhancement.
in the peak corresponding to N1s (400 eV). The quantitative elemental intensity analysis (Table 3.1.1) revealed the success of grafting process of modified Ti with a clear enhancement in the C1s and N1s signals coupled with a decrease in Ti peak intensity. The increase in nitrogen percentage could be due to the presence of N containing amine groups of fibrin and the decrease in Ti peak intensity suggests the complete coverage by fibrin/alginate scaffolding over the metallic surface.

![XPS wide scan spectra](image)

**Figure 3.1.3:** XPS wide scan spectra of (a) control polished Ti (CTi), (b) hydrothermally modified Ti, (c) Dopamine treated Ti and (d) Fibrin/alginate treated Ti (MTi). The Ti 2p and N 1s peaks are represented with a circle and the arrow head represents the absence of Ti 2p peaks in the MTi and dopamine treated Ti plates.

**Table 3.1.1:** Surface elemental intensity of Ti samples detected by XPS

<table>
<thead>
<tr>
<th>Samples</th>
<th>C1s</th>
<th>O1s</th>
<th>N1s</th>
<th>Ti2p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bare Ti</td>
<td>26*10^3</td>
<td>30*10^3</td>
<td>18*10^2</td>
<td>22*10^3</td>
</tr>
<tr>
<td>Hydrothermal Ti</td>
<td>26*10^3</td>
<td>100*10^3</td>
<td>40*10^2</td>
<td>60*10^3</td>
</tr>
<tr>
<td>Dopamine treated</td>
<td>36*10^3</td>
<td>36*10^3</td>
<td>13*10^3</td>
<td>60*10^1</td>
</tr>
<tr>
<td>Fibrin/alginate</td>
<td>60*10^3</td>
<td>70*10^3</td>
<td>22*10^3</td>
<td>32*10^1</td>
</tr>
</tbody>
</table>
3.1.3.4 FTIR Analysis

The FTIR spectra of the samples at different stages of conjugation are depicted in Figure 3.1.4. Hydrothermally treated plates (in NaOH) represented as (ii) in the figure showed two peaks - one corresponding to O-H stretching at ~3400 cm⁻¹ and the other due to O-H bending at ~ 1400 cm⁻¹. This may be due to the formation of Ti-OH groups on the surface of Ti after the high temperature hydrothermal treatment in alkaline NaOH. At high concentrations of aqueous alkali, the general dissolution of Ti species and the re-precipitation of sodium titanate occur as reported by Divya *et al* [11]. The metal surface formed Ti-OH groups by exchanging Na⁺ ions in the surface sodium titanate with H₂O⁺ ions in the water. Dopamine treated plates represented as (iii) revealed a strong infrared peak at ~1650 cm⁻¹ (N-H bending) due to the presence of the amine group of dopamine hydrochloride. The O-H bending peak at 1400 cm⁻¹ is absent in the dopamine treated plates, suggesting the possibility of formation of a bidentate coordination complex between the catechol oxygens of dopamine hydrochloride and Ti atoms as reported elsewhere [214]. Analysis of the FTIR spectra of the composite depicted in (iv) revealed a broadening of the O-H peak ~3500 cm⁻¹ which can be due to the presence of the hydroxyl groups present in alginate. Further, the intensity of the peak at 1650cm⁻¹ was found to be diminished in magnitude, suggesting a possible bonding of fibrin with dopamine. In addition, the characteristic Amide I (~1650 cm⁻¹), Amide II (~1450-1550 cm⁻¹), and Amide III (~1228cm⁻¹) peaks of fibrin are also evident in the composite sample (iv) as shown in the inset of the FTIR spectra. This clearly confirmed the immobilization of fibrin and alginate onto dopamine treated Ti plates.

DOPA containing peptides are widely discussed in literature as intrinsically attractive enediol candidates that can act as bridging ligands. It can react with the hydroxyl groups of Ti and can form ligand to metal charge transfer complexes [159]. Another main advantage of dopamine to be used as an anchor molecule is that, it can form amide bonds with different molecules of interest via the alkyl-NH₂ linkage [215]. In the FTIR analysis of the modified Ti plates, the amide bond (-NHCO-) formed by the reaction between the carboxyl group of the fibrin and amino group of dopamine favoured the bonding of the scaffolding with
the Ti substrate. The FTIR data clearly demonstrated that the immobilization of the scaffold onto Ti is likely to be due to the interaction of dopamine hydrochloride with the fibrin/alginate composite via the bidentate coordination chemistry [216].

![FTIR spectra of the fibrin/alginate scaffolding on Ti by chemical cross linking with dopamine hydrochloride at different steps of conjugation. Inset image shows an enlarged view of the amide peaks of fibrin on modified Ti samples.](image)

**Figure 3.1.4**: FTIR spectra of the fibrin/alginate scaffolding on Ti by chemical cross linking with dopamine hydrochloride at different steps of conjugation. Inset image shows an enlarged view of the amide peaks of fibrin on modified Ti samples.

### 3.1.3.5 PTAH Staining

PTAH is a mixture of two dyes phosphotungstic acid and haematoxylin, which is usually used in histology for staining. PTAH staining, which is a characteristic stain for proteins, specifically fibrin, was used as yet another confirmatory test. PTAH stains fibrin fibers in deep blue. Staining of the fibrin/alginate MTi after PTAH showed the presence of fibrin in the composite layer by the characteristic deep blue colouration of fibrin as shown in Figure 3.1.5
A. On the contrary, the PTAH stained CTi appeared devoid of any stains as shown in Figure 3.1.5 B.

![Image](image.png)

**Figure 3.1.5:** A) PTAH stained MTi plates showing the presence of fibrin in the composite coating by its blue colour and (B) PTAH stained CTi plate.

### 3.1.3.6 Increased Hydrophilicity of the Material

The surface characteristics of a material such as topography, chemistry and surface energy greatly influence the adsorption of biological molecules onto the surface, which in turn aid in better cell attachment. The hydrophilic and hydrophobic characteristics of a material greatly influence the cell adhesion behaviour of the material. It is well established that increased hydrophilicity enhances interaction between implant surfaces and the biological environment [217]. In this study, the surface wettability and surface energy were determined by measuring the contact angles of liquid (water) on the modified and polished Ti plates. The contact angles and surface energy (mJm⁻²) of the substrates are shown in Table 3.1.2. Contact angle for the modified plate was found to be \( \theta = 37.0 \pm 6.88^\circ \), while that of the control polished Ti was \( 62.7 \pm 6.12^\circ \) (Figure 3.1.6). It is evident from other researchers’ results that the mammalian cells can efficiently attach to hydrophilic surfaces in comparison to hydrophobic surfaces [218]. Here, in the present study the surface modification significantly reduced the water contact angle values of modified Ti plates than the control polished Ti. This increased surface wettability or hydrophilicity can be attributed to the hydrophilic nature of both fibrin and alginate coating on the surface of Ti plate. The
alterations in surface topography due to polymer coating also enhanced the surface area of the modified Ti plate that in turn increased the surface free energy of the material, which was calculated using the formula $E_s = E_{iv} \cos \theta$.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Contact angle (°)</th>
<th>Surface Energy (mJ/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Modified Ti</td>
<td>37.0 ± 6.88</td>
<td>58.14 ± 5.2</td>
</tr>
<tr>
<td>Control Ti</td>
<td>62.7 ± 6.12</td>
<td>33.8 ± 6.8</td>
</tr>
</tbody>
</table>

3.1.3.7 Protein Adsorption

Protein adsorption studies are important because any biomaterial immediately after implantation adsorbs serum osteogenic components which can in turn improve the proliferation as well as differentiation ability of different cells, including stem cells and can aid in bone remodelling processes [219]. We have studied the serum protein adsorption behaviour of the modified plates with the polished one as control using BCA assay. There has been a significant increase in protein adsorption on modified Ti than the control Ti plate as shown in Figure 3.1.7. The increased protein adsorption can be attributed to the increased wettability or hydrophilicity of the material. The high surface free energy contributed by nanoscale features on the modified Ti plates could be another
reason for the increased adsorption of serum proteins. Our findings also support other researchers’ findings that surface free energy greatly influences protein adsorption and the structural arrangements of protein moieties on the material [220]. Serum contains multiple cell attachment proteins that are readily adsorbed on implant surface which further determines the cell behaviour on contact such as morphology, proliferation as well as their differentiation. The present in vitro serum adsorption studies are representative of the in vivo situation, wherein the implant material gets covered with various cell attachment proteins such as vitronectin, fibronectin etc. Thus, a detailed study of this specific protein adsorption on the samples should be carried out to get a precise idea of different cell responses to these materials.

Figure 3.1.7: Protein adsorption on modified Ti plates in comparison to a control polished Ti. ** denotes p value <0.01 for n=3 samples.

3.1.3.8 Adhesion Strength Analysis of Polymeric Scaffolding on Ti

Microscratch test was done to analyze the influence of dopamine in the bioscaffolding approach. Dopamine was used as a crosslinker in this scaffolding approach. To analyze the effectiveness of dopamine for crosslinking, we have carried out a microscratch analysis. For the scratch resistance test, the critical load values (Lc) where the delamination of the coating occurred was measured. The critical load where the first cracking (Lc1) and the complete delamination (Lc2) of the coating took place were marked. The average Lc values at which the complete
delamination of the coating occurred for untreated and dopamine treated samples were measured to be 2.54 ± 0.05 N and 4.25 ± 0.29 N respectively.

In the dopamine treated samples, the Lc2 values were substantially enhanced. This enhancement in critical load for delamination can be attributed to the dopamine treatment prior to fibrin/alginate deposition, which provided increased adhesion than untreated samples. Such values of critical load are comparable to the shear force applied during implantation [221]. The results correlated well with the optical microscopic images of the scratches on each sample (Figure 3.1.8). In a similar study with chitosan coating on titanium via silanization, material deformation was reported to start at a critical load value of 5.5 N, which is more or less in the range obtained for the MTi samples in our case [222]. The augmented critical loading resistance of dopamine treated sample signifies the potential of using it as an ideal material for dental/orthopaedic use.

![Image of scratch images](image)

**Figure 3.1.8: Optical microscopic images of scratch observed on microscratch analysis on dopamine untreated sample and dopamine treated sample. The Lc1 and Lc2 values determined from the microscratch test for each of the samples is given.**

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3.2 IN VITRO BIOLOGICAL STUDIES USING hMSCs

The *in vitro* biological studies using hMSCs on the surface modified metallic Ti are addressed in two different sections. In section I, the effect of MTi and CTi on initial cell adhesion and proliferation are discussed and in section II, detailed molecular investigations on the differentiation potential of hMSCs are detailed.

3.2.1 SECTION I- EFFECT ON CELL ADHESION AND PROLIFERATION

3.2.1.1 RESEARCH QUESTION AND HYPOTHESIS

**Question:** How will the bio-polymeric scaffolding influence mesenchymal stem cell (MSC) attachment, proliferation and subsequent differentiation into osteoblast compared to control polished Ti?

**Hypothesis:** Bio-polymeric scaffolding of fibrin-alginate immobilized metallic Ti structurally mimics the ECM of bone, and would provide a more biocompatible environment for MSC adhesion, proliferation and its subsequent differentiation into osteoblasts.

3.2.1.2 RESULTS AND DISCUSSION

3.2.1.3 hMSC Characterization

The hMSCs isolated in our lab from umbilical cord blood showed the characteristic MSC specific surface marker expression of CD-44, CD-73 and CD-29 and were negative for CD-31 (endothelial cells), CD-34 (haematopoietic cells) and CD-33 (myeloid cells) expression (Figure 3.21.1).
Figure 3.2.1.1: A) Schematic representation of mononuclear cell isolation after layering umbilical cord blood over histopaque. Mononuclear cells appear in the interface between histopaque and plasma. (B) Passage 1 hMSCs demonstrating the typical spindle shaped morphology of mesenchymal stem cells. (C) Flow cytometric characterization of the isolated hMSCs from umbilical cord blood. The fluorescent activated cell sorting analysis of hMSCs for CD-44, CD-73 and CD-29 showed positive results; whereas CD-33, CD-34 and CD-31 expressions were negative.

3.2.1.4 SEM and DAPI Analysis

Cell adhesion, spreading and migration on substrates are the first sequential events taking place when coming into contact with a material surface, which is crucial for cell survival. Cell attachment studies were carried out on the Ti plates to evaluate the biocompatibility of the material. Figure 3.2.1.2 shows the morphologies of hMSCs cultured on modified and control Ti at different time intervals. The SEM images and the DAPI staining showed enhanced cell attachment and spreading on the modified Ti plates than the control plates. After 30 min incubation, the cells had a rounded morphology on both the modified and polished plates (Figure 3.2.1.2 a (i)-a (iii)). However, more number of cells was found to adhere on the modified Ti plates than the control Ti. Compared with that
of the control Ti, the cells on modified Ti showed an elongated morphology after 6 h of culture conditions as shown in Figure 3.2.1.2 (biii). The adhered cells developed numerous cellular processes like lamellopodia and filopodia on the modified plates which helped to enhance the cell-substrate as well as cell-cell interactions. The DAPI images also demonstrated the same trend with MTi showing enhanced cell adhesion and proliferation compared to CTi at both the time points.

Figure 3.2.1.2: Representative SEM and DAPI stained images of hMSC attachment on the modified Ti plates and control polished Ti at different time points of incubation is shown; a (i) & b (i) represents SEM and a (ii) and b (ii) represents DAPI images of CTi at 30 min and 6h of incubation; a (iii) & b (iii) represents SEM and a (iv) & b (iv) represents DAPI images of MTi. After 6h, hMSCs started spreading; however cells showed high degree of spreading on modified Ti (white arrow) than control polished Ti plates. In the figure b (iii), the microporous nanofibrous network is also seen which is shown in black arrow.

In our experiment, the shape of the cells on the control and the modified plate appeared to be distinctly different as evident from Fig 3.2.1.2. The cells on surface modified Ti appeared to be more spindle shaped, covering a larger surface area. The cells on the modified Ti covered almost 35 % of the surface area (8000 µm² frame area), compared to 12 % coverage exhibited by the cells on CTi. This can be attributed to the difference in surface topography of the material created by biochemical modification. It is reported that the surface architecture of Ti
substrate can affect the expression of various proteins including fibronectin which is usually responsible for the variations in shape and morphology of cells [223]. The cryoprecipitate, which was used for making fibrin, contains fibronectin and the role of fibronectin in cell adhesion, migration, signalling for proliferation and survival has been well established by various research groups [224]. Since fibrinogen and fibronectin are the major natural components at the sites of vascular injury and angiogenesis, the interaction with these proteins on material surfaces may be a possible reason for better cell proliferation [225].

3.2.1.5 Cytoskeletal Development and Focal Adhesion Protein Expression

Actin organization is an important event in cell adhesion, spreading and its subsequent differentiation. It is a dynamic structure that rapidly changes shape and organization in response to external forces. Therefore any disruption in its function can lead to alterations in cells normal behaviour [226]. The differences in cytoskeletal arrangements on the modified plate in comparison to control Ti plates were evaluated by F-Actin staining as depicted in Figure 3.2.1.3. Confocal microscopic images of hMSCs after anti–actin immunostaining on the modified Ti surfaces exhibited a widespread and dense cytoskeletal arrangement at 2 h (b (i)). After 6 h (b (ii)), the hMSCs on the MTi showed an elongated morphology with several actin stress fibre formation across the cytoplasm. In contrast, the degree of cytoskeletal arrangement on control polished Ti was less organized (a (i) & a (ii)).

Confocal microscopic images (Figure 3.2.1.4) of hMSCs after anti-vinculin staining showed positive localization of vinculin protein on both control Ti as well as modified Ti plates. After 24 h of incubation, the hMSCs on the modified plates showed extensive localization of vinculin around the cell membrane forming well organized focal adhesion points. Whereas, on the control Ti plates, the vinculin expression is more confined to the nuclear region. Also, the lamellopodia formation involved in cellular migration was evident on the modified Ti plate compared to the control polished Ti. Further, the mean fluorescent intensity was quantified using Image J software. Region of interests (ROIs) were selected for each samples to obtain their mean fluorescent intensity
values. The obtained mean fluorescent intensity of modified Ti plate was found to be significantly higher (p<0.05) when compared to control polished Ti. The measured mean fluorescent intensity is represented in a graph below the fluorescent images (Figure 3.2.1.4).

Significantly advanced formation of cytoskeletal arrangement and focal adhesion complexes were observed on the modified Ti than control Ti. The formation of well-defined focal contacts and actin microfilaments on modified Ti surfaces revealed the effective mechanotransduction events occurring on the surfaces which in turn help in the activation of different signalling events promoting cellular adhesion to the different ECM components on the material surface [227]. The synergistic action of actin molecules and the vinculin protein contributed to the enhanced cell adhesion and spreading on modified Ti plates. Moreover, the success of osteointegration depends upon the formation of defined focal adhesion complexes, which include vinculin, talin, integrins, phospholipids and paxillin [228]. Further studies on these interacting molecular events should be carried out to clearly understand the mechanism, whereby the surface topographic cues exerts its effect on cell shape and its further distribution over different study matrices.

Figure 3.2.1.3: Confocal microscopic images of actin stained hMSCs on a (i) & a (ii) control Ti and b (i) & b (ii) on modified Ti at 2 h and 6h of incubation. The interpenetrating network of fibrin/alginate scaffolds can be clearly seen on the modified Ti plate with the cells exhibiting well spread morphology with a highly organized actin cytoskeletal arrangement than the control Ti.
Figure 3.2.1.4: Confocal images of the immunofluorescent staining of vinculin focal adhesion points on control polished Ti and modified Ti at 24 h incubation is shown. Left panel a(i) – a(iii) shows the vinculin focal adhesion studies on control polished Ti and right panel b(i) – b(iii) shows the focal adhesion on modified Ti plates at different magnifications with a(i) and b(i) at 20X magnification, a(ii) and b(ii) 40 X and a(iii) and b(iii) at 63 X respectively. The mean fluorescent intensity quantified on MTi and CTi is represented below (* p<0.05).

3.2.1.6 DNA Quantification Assay

The DNA content compared between control Ti and modified Ti was illustrated in Figure 3.2.1.5. The DNA content on the modified Ti increased over time showing a significant elevation between day 1 and day 4 compared to control polished Ti. Almost 10 fold increase in DNA content noticed for MTi on 4th day with that of 1st day. However, only a 2 fold increase in DNA content was observed on the control polished Ti between 1st and 4th days. These results collectively
suggest that the modified Ti supported enhanced cell proliferation than the unmodified Ti. Here, we observed excellent cell adhesion and proliferation of hMSCs on the modified surfaces signifying the nanofibrous network of fibrin aiding cell-material interaction. The effect of fibrin on cell proliferation was well demonstrated in a wide variety of cell types including osteoblasts, periodontal ligament cells, gingival fibroblasts, oral epithelial cells, BMSCs, pre-adipocytes, and pre-keratinocytes [229]. Again, a similar trend of cellular proliferation was observed on a study wherein, fibrin glue coated bioactive glass ceramics enhanced the proliferation and differentiation of goat bone marrow derived stem cells [230]. All these facts point to the benefits of using fibrin as an extracellular matrix for stem cell proliferation and its subsequent differentiation. Alginate on the other hand, due to lack of specific cell adhesion sequences provided the structural integrity to the polymeric scaffolding rather than playing its role in cell adhesion and its proliferation [231, 232].

![Graphical representation of the DNA quantification by picogreen dsDNA analysis of the cells grown on control and modified Ti plates at 1st and 4th days. Cell number calculated is shown in inset. The higher DNA content on the modified Ti compared to the control polished Ti was statistically significant with p<0.01 (n=3).](image)

**Figure 3.2.1.5: Graphical representation of the DNA quantification by picogreen dsDNA analysis of the cells grown on control and modified Ti plates at 1st and 4th days. Cell number calculated is shown in inset. The higher DNA content on the modified Ti compared to the control polished Ti was statistically significant with p<0.01 (n=3).**

### 3.2.1.7 Cell Viability Assay

hMSCs viability on the control as well as modified Ti plates were evaluated using live/dead assay. Figure 3.2.1.6 shows the fluorescent microscopic images of the plates stained with calcein AM and Ethidium homodimer- 1. Live
cells appeared green and dead cells appear red in colour. The analysis showed that both the control as well as modified Ti plates favoured cell adhesion with the latter showing improved cell survival. In the modified Ti plates, a significantly higher percentage of viable cells were detected whereas, in the control Ti plates a few dead cell cells were seen. One main requirement for the osteointegration of the metallic implants is the ability to support the initial adhesion of osteoprogenitors to the scaffold surface during early stages of implantation. The adhered cells should be viable so that it can differentiate into functional osteoblasts and finally produce mineralized matrix [233]. The viability of a cell on a material depends on multitude of factors. Substrate composition is one main factor which favours the integrin specific binding of cells and its subsequent spreading [234]. Proper cellular spreading helps in achieving cell-cell contact, which activates the signalling events responsible for maintaining the cell survival and its further growth. Here, the fibrin scaffolding over Ti was found to favour the adhesion, spreading and survival of human mesenchymal stem cells than control polished Ti surfaces. The ability of fibrin gels to act as a temporary matrix for cellular survival was demonstrated in various studies [235, 236]. With the aid of RGD motifs, it can bind to the cells surface receptors, mainly integrins and can initiate the signalling cascade mediating cell survival [237].

![Fluorescent microscopic images showing hMSCs viability by live dead assay on study matrices (a) control polished Ti and (b) modified Ti. Calcein AM stains live cells in green and Ethidium homodimer stains dead cells in red.](image)

**Figure 3.2.1.6:** Fluorescent microscopic images showing hMSCs viability by live dead assay on study matrices (a) control polished Ti and (b) modified Ti. Calcein AM stains live cells in green and Ethidium homodimer stains dead cells in red.
3.2.1.8 Apoptosis Studies

The apoptotic assay was also carried out to quantify the percentage of cellular viability using flow cytometry. Annexin V-FITC was used as a positive marker, which can identify the apoptotic cells by binding to the phosphatidyl serine moiety exposed on the outer leaflet of plasma membrane during apoptosis and Propidium Iodide (PI) was used for detecting the necrotic cells. The results were compared with a negative control, in which the cells were grown on polystyrene culture plate. The percentage of cells that underwent apoptosis is represented in boxes as P2 population. Figure 3.2.1.7 represents the flow cytogram of FITC-Annexin-V/ PI staining of hMSCs grown on modified Ti surfaces and control polished Ti in comparison to a negative control. Our results indicated that the modified Ti plate did not induce significant rate of apoptosis or necrosis of hMSCs when compared to a control Ti plate and negative control even after 72 h of incubation. Also, the modified Ti plates exhibited above 90% cell viability which also matches up with the results of Live/Dead assay. The percentage viability was found to be 97 % for cells on MTi and 40 % for cells cultured on CTi.

Figure 3.2.1.7: Annexin V apoptotic binding assay of hMSCs on (a) negative control (cells grown on the polystyrene culture plate) (b) on modified Ti plates and (c) on control polished Ti; with P2 population shown in boxes representing the percentage of apoptotic cells.

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3.2.2 SECTION II- EFFECT ON BONE MATRIX PROTEIN EXPRESSION

3.2.2.1 RESEARCH QUESTION AND HYPOTHESIS

**Question:** What will be the effect of modified Ti substrates on the expression of bone specific matrix proteins compared to control polished Ti?

**Hypothesis:** Bio-polymeric scaffolding approach on titanium implants can enhance osteoblast-specific gene expression at the transcriptional level due to the changes in the mechanotransduction events and can facilitate the production of specific matrix proteins than unmodified Ti substrates.

3.2.2.2 RESULTS AND DISCUSSION

3.2.2.3 ALP Activity

The ALP activity assessed on CTi and MTi for a period of 28 days showed a typical trend in increase in ALP activity followed by a dip from 28 days onwards. As ALP activity is a widely used marker for evaluating stem cell differentiation into osteoblastic lineage, the results (Figure 3.2.2.1) showed that the osteogenic induced hMSCs expressed a sequential increase in ALP activity both in CTi and MTi, confirming its differentiation, with MTi showing a 1.5 fold increase in ALP activity than CTi. The highest ALP activity was observed on 21st day, followed by a decline in activity from 28th day onwards. It is well reported that the ALP activity peaks during the osteoblastic differentiation stage and when the osteoblasts enters into the stage of maturation, the activity reduces. The enhanced ALP activity of hMSCs on MTi could be due to the presence of osteogenic as well as angiogenic factor fibrin [238]. The role of fibrin in mediating the differentiation of mesenchymal stem cells into osteogenic lineage was well projected in many studies [239-241]. Moreover, the presence of RGD and AGDV sequence in fibrin, where the cellular integrins bind also might have aided in the enhanced differentiation of hMSCs to osteoblast [242, 243].
Chapter 3  Results and Discussion

3.2.2.4 Gene Expression Analysis

Since the surface properties of the implant could greatly influence the regulation of osteo-specific genes, gene expression studies often reveal the behaviour of critical up- or down-regulation of specific genes involved in the osteogenesis, when in contact with the study matrices. The enhanced expression of certain genes such as ALP, OC, OPN and COL I at the implant – bone interface will promote early osteointegration [210]. Hence, to elucidate the molecular mechanism behind the osteoblastic differentiation of hMSCs, mRNA level expressions of osteoblastic genes such as ALP, COL I, OPN and OC was analyzed for a period of 28 days. The culture was stopped at definite time intervals and the mRNA level of target genes was analyzed. Figure 3.2.2.2 reveals significant changes in the fold expression of all genes on MTi compared to CTi. The ALP expression on MTi was almost 20 fold up-regulated at day 7 than CTi, with a gradual decrease in expression noticed by day 28. The data supported the fact that ALP is an early osteoblastic differentiation marker whose expression is high during the early phases of bone development and tends to decline when osteoblasts enter the maturation phase [244]. Similarly, COL I, OC and OPN expressions were also high on MTi, with collagen showing a ~15 fold increase in expression on day 28 than CTi. OC and OPN expression levels showed a higher
fold increase (~75 & 65) on day 28 than day 7 on the bio-polymeric scaffolded Ti compared to CTi. This is justifiable because both the genes OC and OPN are involved in the late stage maturation of bone matrix [191].

Figure 3.2.2.2: Real Time PCR analysis showing the fold increase in mRNA level expression of osteoblast specific genes; ALP, COL I, BGLAP (OC) and SPP 1 (OPN). Apart from ALP, all other genes showing highest expression on 28th day with respect to CTi. All data points represented were normalized with that of the reference gene β-Actin.

This temporal up-regulation pattern in gene expression on MTi can be attributed to the presence of fibrin. Several studies have shown the evidence of fibrin in inducing osteogenic differentiation of mesenchymal stem cells into differentiated osteoblasts [245]. One of the reasons reported for the increased cellular attachment, proliferation and differentiation of stem cells on fibrin matrices is the presence of the binding sites for αvβ3 and αvβ1 integrin receptors which favour cell binding [246]. Abiraman et al [131] has also demonstrated the osteoinductive nature of fibrin glue when implanted in the extraskeletal site of swiss albino mice. A similar trend in fold increase in ALP, OC and OPN was also noticed in a study with fibrin glue coated ceramic scaffolds [247]. One of the major criteria for bone formation is the ability of osteoblast cells to secrete and mineralize the matrix through the synthesis of bone-specific extracellular matrix proteins such as ALP, COL I, OPN, OC, etc [248]. This synthesis mechanism is favoured mainly due to the up-regulation of certain transcription factors such as
RUNX2, osterix, etc., which regulate skeletogenesis by mediating other target genes such as COL I, OC and OPN [249]. Possible mechanism for the accelerated expression of osteoblasts markers on MTi compared to CTi include multiple cross talks mediated by the RUNX2 over expression triggering the downstream osteogenic signalling cascade, consequently results in the enhanced ALP, OC, OPN and COL I synthesis.

3.2.2.5 Immunofluorescence Analysis

Immunofluorescence analysis of bone-specific matrix protein expression was carried out to corroborate the differentiation of hMSCs to osteoblasts. Differentiating osteoblasts are very well known for their ability to synthesize matrix components such as ALP, COL I and non-collagenous matrix proteins such as OPN, OC, osteonectin, bone sialoprotein (BSP), etc. [250, 251]. Of these proteins, the expression of ALP, Col I, OPN and OC were analyzed in addition to evaluating the expression of RUNX2, which is an early bone transcription factor. As evident from Figure 3.2.2.3 (a), the cells on both CTi and MTi expressed positive immunolabelling for RUNX2 antibody at 7 and 28 days, with a noticeable increase detected on MTi.

![Figure 3.2.2.3](image)

*Figure 3.2.2.3: a) Fluorescent microscopic images of differentiated hMSCs showing RUNX2 expression on MTi and CTi. Cells were stained with anti- RUNX2 antibody (green, scale bar representing 50µm).*
The enhancement in RUNX2 expression was also evident from FACS analysis (Figure 3.2.2.3 b and c). RUNX2 is one of the early key transcription factors which determine the fate of MSCs to go into osteoblastic lineage. It enhances the differentiation of multipotent stem cells into osteoblasts at an early stage [252-254]. The down regulation of RUNX2 expression after 28 days of culture was found to be sharper on MTi than CTi, which suggested that the cells differentiated more effectively and produced other matrix components such as COLI, ALP, OPN and OC.

![Figure 3.2.2.3: (b) represents the flow cytometric quantification of RUNX2 expression on CTi (ai &aii) and MTi (bi & bii) for 7 and 28 days with; P2 population representing percentage of RUNX2 expression. Percentage expression of RUNX2 by differentiated hMSCs on MTi and CTi is plotted separately and represented in c. *p<0.05.](image)

The expression of Type I collagen after 7 and 28 days of culture, correlated well with this finding, which is represented in Fig. 3.2.2.4. Cells grown on MTi showed a positive staining of Col I at 7 days of culture, while no collagenous matrix deposition was observed on CTi at this time point. Moreover, an increased COL I deposition was noted on MTi at 28 days, which was significantly higher than on CTi and more uniformly distributed as well. Type I collagen in the major component of bone extracellular matrix proteins which is recognized as one of the important osteogenic markers and act as the nucleation site for mineralization [117]. Thus, enhanced expression of Type I collagen is always an essential requirement for matrix production and subsequent mineralization [255].
Figure 3.2.2.4: Fluorescent microscopic images of differentiated hMSCs showing Collagen type I (COL I) expression on MTi and CTi at two different magnifications (a & b). Cells were stained with anti COL I antibody (green) for collagen fibrils and Sytox orange for nucleus (red). Scale bar representing 50µm. The mean fluorescent intensity of COL I obtained by Image J analysis is represented in (c). MTi showing high fluorescent intensity compared to CTi on both time periods. Data is represented as mean ± standard deviation. ** represents p<0.01.

The differentiation of hMSCs into osteogenic lineage was further substantiated by the expression of ALP, OPN and OC. ALP is an early marker of osteoblastic differentiation which then gets down regulated during the maturation process [256], while OPN and OC are the two non-collagenous matrix proteins categorized as late stage osteoblastic markers. As evident from Figure 3.2.2.5, MTi showed an upregulated expression of ALP on day 7 than CTi, which clearly indicated that the hMSCs entered into the differentiation phase much earlier. OPN is a mature osteoblastic marker and OC is a marker of fully differentiated osteoblastic cells which play an important role in matrix mineralization [257-259]. The expression levels of OC and OPN were minimal on both MTi and CTi on day...
7, indicating that the cells are not differentiated to osteoblasts. After 28 days, hMSCs grown on MTi showed a sharp increase in the expression of OC and OPN compared to CTi. The enhanced expression of all these osteoblastic markers on MTi could be attributed to the presence of fibrin, which is an established osteogenic as well as angiogenic factor [131]. The role of fibrin in mediating the differentiation of mesenchymal stem cells into osteogenic lineage was well projected in several studies [128, 257]. The presence of cell recognition sequence RGD present on fibrin might have triggered the signalling events mediated by integrins, actin stress fibres and focal adhesion complexes, which in turn could have altered the gene expression profile. Advanced cytoskeletal and matured focal adhesion complex formation were observed on MTi plates whereas, cells grown on CTi failed to form such focal adhesion points. Hence we believe that the physical stresses from the material surface might have activated the mechanotransduction events on MTi substrates which in turn stimulated the RUNX2 signalling cascade, leading to the up-regulated expression of osteogenic factors.

![Figure 3.2.2.5: Fluorescent micrographs showing the immunocytochemical staining of 7th and 28th day expression of bone matrix components OC, OPN and ALP on MTi and CTi (scale bar 10 µm). The expression of OC and OPN got enhanced on MTi with ALP showing reduction in expression compared to day 7. Actin filaments were stained with Texas red phalliodin (red), cell nuclei with DAPI (blue) and OC, OPN and ALP with Alexa Fluor conjugated specific antibodies (green).](image-url)
3.2.2.6 Masson’s Trichrome Staining for Collagenous Matrix Deposition

Trichrome staining is an additional test to confirm the deposition of fibrillar collagenous matrix by the cells. In accordance with the immunofluorescent data, on day 7 minimal collagen deposition was observed on MTi in comparison to CTi where no deposition occurred. After 28 days, the MTi substrate became fully stained blue with fibrillar collagen deposition. Meagre blue coloration was also observed on CTi plates (Figure 3.2.2.6). Since fibrillar collagen deposition is one prerequisite factor for the biomineralization to instigate, the significant enhancement of COL I expression on MTi demonstrated the effectiveness of the fibrin/alginate matrix in aiding the differentiation and maturation of osteoblasts. Also, there was evidence of increased production of Collagen type I by fibroblast, endothelial cells and smooth muscle cells when they were entrapped in fibrin gels [191, 245].

![Figure 3.2.2.6.TRICHROME staining of collagenous matrix deposition on MTi and CTi after 7th and 28th days of culture. Trichrome stains both collagen (blue; black arrows) and fibrin (red). Enhanced collagen production on MTi is evident from figure.](image-url)
3.2.2.7 In vitro Mineralization- Acellular Method

The *in vitro* apatite forming ability assessed by acellular method showed enhanced calcium phosphate deposition on modified Ti than control Ti surfaces. The surface of each Ti plates after immersion in SBF is shown in Figure 3.2.2.7 (A). The apatite deposit could be seen on the modified as well as the control plates on 3rd day onwards, but the number of calcium phosphate spherulite was found to be more on modified Ti than control polished Ti. The elementary composition of calcium and phosphate deposits analyzed with the EDAX attachment of SEM (Figure 3.2.2.7 (B)) gave the calcium phosphate ratios ranging from 1.63-1.86. which correspond to the stochiometric ratio of hydroxyapatite [260].

![Figure 3.2.2.7: A) SEM photographs showing the surfaces of each Ti plates after mineralization in 1.5X SBF and B; the respective EDAX data of the mineralization from day1 to day7.](image)

3.2.2.8 In vitro Mineralization-Cellular Method

Mineralization ability of osteogenically induced hMSCs on the modified Ti and control Ti plates was measured at different time intervals from day 7 to day 21. SEM images (Figure 3.2.2.8 a) showed the presence of calcium deposits on modified Ti plates by day 21 with a nearly complete coverage of mineralized...
matrix over the surface. On the other hand, the hMSCs seeded on to the control Ti showed less mineral deposition compared to modified Ti. The deposited minerals on modified Ti were confirmed to be that of calcium and phosphorous with a Ca/P ratio of 1.8 similar to that of native bony apatite (Figure 3.2.2.8 b).

![Figure 3.2.2.8: (a) SEM images showing the biomineralization of hMSCs on modified Ti plates and control polished plates analyzed for a period of 21 days, (b) the respective EDAX data of the mineralized modified Ti plates on 21 day shows the calcium phosphate ratio of 1.8.]

### 3.2.2.9 Alizarin Red S Staining

Alizarin Red S staining, which is a specific stain used to identify the presence of calcific deposition by cells of an osteogenic lineage. Alizarin Red staining carried out on the different Ti substrates confirmed the osteogenic differentiation of hMSCs. Cells plated on the modified Ti showed a substantial level of staining compared to control Ti plates. By day 14, the cells on the modified Ti showed positive staining for Alizarin Red S. The intensity of the stain got enhanced by day 21. Whereas, the cells plated on control polished Ti showed only a modest staining (Figure 3.2.2.9).
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Figure 3.2.2.9: Optical microscopic images of the Alizarin red stained hMSCs on control Ti and modified Ti on 7, 14 and 21 days. Alizarin stained plates are shown in insets. The orange-red colouration of calcium deposits is evident on the modified plates.

The *in vitro* mineralization ability of the Ti substrates by both the acellular and cellular means demonstrated the same trend of enhanced calcium phosphate deposition on the modified Ti than the control Ti. These findings support the results of Kokubo *et al* [261] that an essential requirement for *in vivo* bone growth on a synthetic material is the formation of a calcium phosphate layer on the material surface, usually called bone-like apatite which in turn triggers a cascade of events resulting in bone formation.

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3.3 BLOOD-MATERIAL INTERACTION STUDIES

3.3.1 INTRODUCTION

The success of an intramedullary implant mainly depends on its ability to integrate with host bone tissue via osteointegration [262]. A multitude of factors play a crucial role in favouring proper integration between living bone and the surface of a load bearing Ti implant. Of these, surface topography of implants plays a significant role [263]. One of the decisive factors reported to be responsible for accelerating the integration process is the interaction of endoprosthesis with blood components [264]. In intramedullary implantation, blood and its components first come in contact with the foreign material that is implanted [265]. It is reported that the blood components, mainly the platelets, promote blood clotting and release factors responsible for the osseointegration of implant materials. Both blood clotting and platelet activation were found to influence the stem cell recruitment and subsequent matrix deposition [266]. Reports suggest that implants with different surface textures modulate platelet activity to a great extent. Recently Milleret et al [267] reported on the effect of micro-rough titanium surfaces in platelet activation and macrophage/monocyte adhesion. Different nanomodified Ti implants were also found to enhance platelet activation and aggregation when compared to polished Ti surfaces [268, 269]. Since the nature of the interactions between blood and endosseous implants influences subsequent bone healing events, it is indispensable to monitor the changes in blood components when coming into contact with the biomaterial. Here, we have analyzed the effect of our bio-polymeric scaffold on Ti in inducing hemolysis, platelet adhesion and activation as well as its influence on the coagulation cascade, compared to an unmodified Ti surface.
3.3.2 RESEARCH QUESTION AND HYPOTHESIS

**Question**: What will be the influence of the surface modification of Ti on interactions with blood components?

**Hypothesis**: Fibrin being a natural scaffold formed during wound healing process, polymeric scaffolding made of fibrin can help in inducing platelet activation at a basal level releasing mitogenic factors for MSCs without compromising the hemocompatibility of the material.

3.3.3 RESULTS AND DISCUSSION

3.3.3.1 Hemolytic Potential

The impact of CTi and MTi on erythrocytes membrane integrity was analyzed based on the soret band based analysis of free hemoglobin present in blood plasma. Figure 3.3.1 shows the % hemolysis measured on samples kept in contact with blood for 1 hour. The results were compared with a positive control Triton X-100 and negative control PBS. The % hemolysis of CTi and MTi were comparable with that of negative control. The corresponding digital images of the four samples, viz., positive control, negative control, CTi and MTi, revealed the absence of any hemolysis in the samples, while Triton treated blood showed ~100% lysis of RBC. This was evident from the red colour of the supernatant caused by the release of hemoglobin into the plasma. SEM images were also taken to clearly understand the RBC membrane integrity from which it was clear that CTi and MTi did not alter the membrane integrity of erythrocytes (Figure 3.3.1 (b (i) & b (ii)). Loss of membrane integrity of erythrocytes ultimately resulted in its break down which would directly affect the circulatory system transporting oxygen to body tissues [270]. A less hemolysis ratio indicates good hemocompatibility and the potential of the material to be used as interfaces for medically implantable devices.
3.3.3.2 Platelet Adhesion and Activation

Immediately upon orthopaedic prosthesis insertion into the body, platelets are the first cell types which come into contact with the implant surface. Its adhesion followed by activation is an important event triggering many intracellular processes such as recruitment of monocytes, neutrophils, and mesenchymal cells toward the implant surface [271]. Hence the effect of biopolymeric scaffolding approach on platelet adhesion and activation was also carried out. The SEM images (Figure 3.3.2) revealed a high platelet adhesion on MTi compared to CTi. But the adhered platelets did not form aggregates as in the case of collagen coated cover slips, which is known to be a positive activator of platelets [272]. The platelets on MTi were found activated, with pseudopodial extensions clearly visible from the high magnification inset image.
Figure 3.3.2: Representative SEM images showing adhered platelets on (a) MTi, (b) CTi and (c) Collagen coated plates. High magnification images shown in insets depict the morphology of adhered platelets with pseudopodial extension, evident on MTi.

The percentage of activated platelets on CTi and MTi was also quantitatively analyzed by flow cytometry, based on the expression of GP1b-IX (CD-42b), which is present on activated as well as resting platelets (representing the Q4 quadrant) and P-selectin which is secreted only by the activated platelets (representing Q2 quadrant). From the results (Figure 3.3.3), it is evident that there was increased platelet activation on MTi (~30%) than CTi. During platelet activation, the unactivated platelets which appear as compact round bodies will produce pseudopodial extensions and become stellate shaped structures, resulting in the release of several growth factors which act as chemotactic agents for MSC progenitors and aid in wound healing and matrix deposition [273]. In the collagen treated samples, which is the positive control, ~85% of the platelets were showing activation while in the negative control only 13% of activation was observed.
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Figure 3.3.3: FACS analysis showing platelet activation of cells treated with (a) collagen (positive control), (b) PRP isolated (negative control), (c) MTi and (d) CTi. Mild activation (~ 30%) is shown by platelets exposed to MTi compared to positive control where almost 85% of platelets are over-expressing activated platelet protein CD 62P.

The platelet activation was further confirmed by confocal microscopic analysis. Collagen treated samples showed only red fluorescence, indicating the expression of CD 62P (activated platelets), while in the negative control and CTi, only green colour fluorescence was observed, which corresponds to the resting platelets expressing only CD42b. On the contrary, on MTi, a mix of both green and moderate red fluorescence was observed, signifying mild activation of platelets (Figure 3.3.4). As mentioned earlier, platelets are of profound importance in bone formation, as its activation results in the release of cytokines and growth factors that are known to accelerate bone healing [274]. In normal bone injury, de novo bone formation relies on the migration of osteoprogenitors through the provisional fibrin matrix formed. The initial adhesion of platelets to the fibrin is mediated by the platelet glycoprotein, and its activation results in cellular rearrangement, resulting in the release of storage granules into the extracellular matrix environment. These include platelet derived growth factor (PDGF), transforming growth factor (TGF-β) and vasoactive factors such as serotonin and histamine [275, 276]. This provisional fibrin matrix formed at the healing site is favouring subsequent osteogenesis [277]. The implant surface topography plays a
crucial role in modulating the platelet activation and subsequent cellular migration towards the surface of implants [278]. Herein, the surface scaffolding fabricated on MTi includes fibrin as one of the polymeric components, whose interaction with the platelets might have contributed to the enhanced activation compared to CTi. The platelet activation potential of thrombin as well as calcium is also well highlighted in literature [279, 280]. Here, during the processing step of MTi, thrombin in CaCl₂ was utilized for the fabrication of fibrin/alginate matrix; the presence of which might have also contributed to the increased platelet activation. Since platelet activation is an important event triggering many intracellular processes such as recruitment of many cell types to the defective site, thereby resulting in osteogenesis, mild activation shown by MTi suggests it favourable use as interfaces for orthopaedic implants.

![Confocal microscopic images showing platelet activation](image)

**Figure 3.3.4:** Confocal microscopic images showing platelet activation on a) collagen treated plate (Positive control), b) Negative control (platelet rich plasma), c) Platelet exposed MTi plates and d) platelet exposed CTi plates. Collagen treated plates and MTi plates showing red fluorescence representative of PE-Cy5 conjugated CD 62 P (p-selectin) expression; whereas negative control and CTi only green fluorescence of FITC conjugated CD42b is seen demonstrating no platelet activation.
3.3.3.3. Analysis of Coagulation Time

An analysis of the effect of study matrices on the clotting kinetics was also carried out by surveying the two main coagulation pathways, viz., intrinsic and extrinsic. APTT (activated partial thromboplastin time) assay measures activation of intrinsic coagulation factors such as factor XII, prekallikrein, highmolecular weight kininogen, factors XI, IX, VIII, X, V, II and fibrinogen and PT (prothrombin time) measures the activation of extrinsic coagulation factors such as VII, X, V, II and fibrinogen. Two time points were analyzed, immediately after contact with material and 1 hour after contact. Although APTT results (Figure 3.3.5 (a) showed a slight decrease in clotting time of MTi compared to CTi and negative control, the change was not that statistically significant. PT measurements (Figure 3.3.5 (b) also yielded values that were within the normal range of 11-15 sec, implying no change in clotting factors on any of the samples.

Blood coagulation properties of implantable materials is imperative, as it can result in impaired wound healing, followed by impaired implant integration [265].

![Graphs representing APTT and PT values](image)

*Figure 3.3.5: Graphs representing the APTT (a) and PT (b) values of blood plasma after 5 minutes and 1 hour of exposure. No interference in APTT and PT values noticed on CTi and MTi, both fell well within the normal range (box area) as that of blood plasma (Negative control (NC)).*
The platelet activation and coagulation pathways are two interdependent processes. The platelets usually interact with many of the coagulation factors and activate the coagulation cascade [281]. Though the platelet activation was found to be higher on MTi, the coagulation cascade was not found to be altered to a greater extent. The slightly lesser time noted for the APTT results on the MTi plates, though it is not highly significant, could be the action of fibrin, which can activate the coagulation cascade and produce the clot formation. Formation of a stable clot is required for osteoconduction [282]. Since the prolongation of clotting time can hamper with the healing time, it is mandatory to test the influence of endoprosthetic devices on clotting time. Moreover, we have utilized bovine thrombin for fabricating the fibrin/alginate scaffold. It also demanded the plasma clotting time analysis, because of the reported coagulopathies associated with use of bovine thrombin [283]. In addition, Ca^{2+} ions also play an important role in the activation of various coagulation factors such as FIX, FX, FII etc [284]. Since the APTT and PT values were well within the normal range, no interference of coagulation cascade was observed.

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3.4 IN VIVO OSTEOINTEGRATION STUDIES IN NEWZEALAND WHITE RABBITS

3.4.1 INTRODUCTION

The success of orthopaedic and dental implants depends mainly on the development of a stable bone-implant interface [285]. Even though artificial joint replacements can function properly for a decade, the long term success of arthroplasties is often limited by implant loosening and wear. Osteointegration, which quantifies the level of bone-implant contact, is highly dependent on the implant surface topography [286]. If an implant with poor compatibility is inserted into the body, undesirable host response such as foreign body reaction and fibrous tissue encapsulation can take place around the implant. Fibrous tissue formation is found to be one of the main reasons for loosening of implants from the bone tissue, leading to implant failure [287]. Considerable efforts are being taken nowadays to improve the life expectancy of clinically available implants. These include modifying the surface topography using physical, chemical and biochemical means [43]. Several studies have shown how surface engineering can aid in direct interaction of osteoblasts with the implant surface and enhance osteointegration [288-290]. Biologically modified surfaces have revealed a positive interaction with osteoblasts and promoted enhanced osteointegration [291-293].

The in vitro studies performed using surface modified Ti developed in this work have demonstrated an enhanced mesenchymal stem cell attachment, proliferation and its subsequent differentiation into bone forming cells, osteoblasts compared to control polished Ti. To further elucidate the potential of this biopolymeric scaffolding approach on the bone bonding ability and new bone formation, suitable animal studies are essential. This part of the thesis work is focused on a detailed investigation of the in vivo bone bonding capability of three different Ti substrates: (i) CTi, (ii) modified Ti implants MTi (without cells) and (iii) in vitro developed surface bony layer (Ti with osteogenically induced MSCs) in a rabbit intramedullary model. For developing the third substrate, rabbit adipose derived MSCs were isolated to generate bone layer on modified Ti implants to avoid the chance of immune rejection.
3.4.2 RESEARCH QUESTION AND HYPOTHESIS

**Question:** To what extent does the bio-polymeric scaffolding approach and the *in vitro* developed surface bony layer on Ti implant help to improve the implant tissue integration compared to control polished Ti in a rabbit model?

**Hypothesis:** The bio-polymeric modification approach on Ti implants and the generation of a surface bony layer on modified Ti implants would aid in enhanced bone apposition and thereby significantly improve functional implant osteointegration compared to a control polished Ti substrate.

3.4.3 RESULTS AND DISCUSSION

3.4.3.1 Bio-polymeric Scaffolding on Ti Rods

For *in vivo* implantation studies Ti cylindrical rods were custom made as per ISO standards (ISO 10993-6:2007). Ti rods of 2 mm diameter and 6 mm length were employed for the study. The bio-polymeric scaffolding was done on Ti rods utilizing fibrin and alginate *via* dopamine linkage. The as formed scaffolding demonstrated a porous architecture which was clearly visible from SEM as well as fluorescent microscopic observation (Figure 3.4.1).

![Figure 3.4.1: SEM image of the custom made Ti rod (A), polished surface of CTi rod (B), Fibrin/alginate scaffolding over MTi (C) and fluorescent microscopic image of the interpenetrating network of fibrin/alginate on MTi (D).]
3.4.3.2 Rabbit ADSC Isolation and Flow Cytometric Evaluation of MSC Markers

ADSCs isolated after collagenase treatment from inguinal adipose tissue of New Zealand White rabbits formed colonies within 2-3 days of culturing. The cells demonstrated the typical spindle shaped morphology of mesenchymal stem cells as shown in Figure 3.4.2.

![Figure 3.4.2: Isolation of adipose tissue from inguinal fat pads (b) of New Zealand white rabbits (a). The collected tissue in antibiotic containing media (c) and (d) the isolated ADSCs showing the typical spindle shaped morphology of MSCs.]

The isolated rabbit ADSCs were positive for characteristic MSC specific CD90 expression (Figure 3.4.3). Almost 90 % of the isolated cells showed positive CD90 expression.

![Figure 3.4.3: FACS analysis of isolated ADSCs. (a) Flow cytogram of unstained ADSCs, (b) Passage 2 ADSCs showing positive CD-90 expression.]

3.4.3.3 Trilineage Differentiation Studies

As proposed by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy, MSCs should have the ability to differentiate into multiple cell lineages such as osteoblasts, chondrocytes and adipocytes. The trilineage potential of isolated ADSCs was analyzed biochemically after inducing them with appropriate inductions factors. Culture expanded ADSCs differentiated well into adipogenic, chondrogenic and osteogenic lineages. In the adipogenic induced ADSC culture, formation of oil droplets could be seen after 28 days of induction (Figure 3.4.4). The oil droplets stained reddish in colour after staining with Oil Red O. Oil Red O is a lysochrome diazo dye commonly used for staining triglycerides and lipids. It mainly targets fat deposits and provides a deep red colour for the fat globules.

![Figure 3.4.4: Adipogenic differentiation of ADSCs. The phase contrast microscopic images of the differentiated ADSCs showing characteristic oil droplets appearance of adipocytes (a) and (b) Positive Oil red O stained images of differentiated ADSCs.](image)

The chondrogenic differentiation is analyzed by staining the chondrogenic induced ADSCs with Safranin O. Safranin O is commonly used for the detection of cartilage tissue. It which gives off a reddish orange appearance for the glycosaminoglycan deposits (Figure 3.4.5).
Figure 3.4.5: Chondrogenic differentiation of ADSCs. The phase contrast microscopic images of the differentiated ADSCs showing characteristic round appearance of chondrocytes (a) and (b) SafraninO stained images of differentiated ADSCs.

The osteogenic induced MSCs showed mineralization of matrix after 28 days of culture conditions as shown in Figure 3.4.6. The matrix stained red after Alizarin Red S staining, which specifically stains the calcium nodules.

Figure 3.4.6: Osteoblastic differentiation of ADSCs. The phase contrast microscopic images of the differentiated ADSCs showing characteristic concentric lamellar formation of osteoblasts (a) and (b) Positive Alizarin red S stained images of differentiated ADSCs.

The multilineage potential of the ADSCs analyzed demonstrated that the isolated cells do possess the characteristics of mesenchymal stem cells. After appropriate culture induction, the isolated cells differentiated into adipogenic, chondrogenic and osteogenic lineages. ADSCs are widely utilized nowadays as an alternating source of MSCs for bone tissue engineering applications. For instance, Hicok et al [294] demonstrated that ADSCs are capable of forming bone in vivo
when they were loaded onto hydroxyapatite/tricalcium phosphate (HA-TPC) matrices. Cowan et al. also showed in his mouse experiments that ADSCs purified from rodent’s belly fat were able to mineralize bone in a skull fracture site [295]. Because of its potential to differentiate into osteoblastic lineage, adipose derived stem cells are considered nowadays as an alternative prospective source of stem cells that could have far reaching effects on several fields including bone engineering.

### 3.4.3.4 Live/Dead Assay

The viability of isolated rabbit ADSCs on MTi and CTi were analyzed by staining the cell seeded MTi and CTi with Calcein AM and Ethidium homodimer. Calcein AM stains live cells green in colour and Ethidium homodimer stains dead cells in red. As evident from Figure 3.4.7, ADSCs on MTi demonstrated enhanced viability after 24 h compared to CTi. The MTi plates were fully covered with viable cells and no dead cells were noticed whereas, on CTi very few viable cells were seen. Similar to what observed with hMSCs, ADSCs were found to be well attached and adhered onto MTi. Appropriate cues from the substrate are needed to develop proper cell-material interaction and cell-cell contact, which ultimately results in long term cell survival. Here, the bio-polymeric scaffolding approach is favouring the ADSC viability in an analogous manner as that of hMSCs.

![Figure 3.4.7: Fluorescent microscopic images of the live/dead staining of ADSCs on Ti rods after 24 h incubation. Compared to CTi (a), ADSCs on MTi showed enhanced viability and cell adhesion (b).](image)

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3.4.3.5 DNA Quantification

Picogreen DNA Quantification assay was done to assess the DNA content of ADSCs on MTi and CTi. The DNA content compared between MTi and CTi was illustrated in Figure 3.4.8. The DNA content of ADSCs on MTi got increased after 4 days compared to CTi. Almost 2 fold increase in DNA concentration was observed on MTi on day 4 compared to CTi. Our results are in consistent with that of our previous study with hMSCs, wherein improved cellular proliferation was noticed on MTi. The modification strategy is indeed helpful in improving cell growth and tissue formation. The improved receptor recognition of fibrillar fibrin has been reported to enhance stem cell proliferation and its subsequent differentiation [296].

![Figure 3.4.8: Picogreen DNA quantification for analyzing the proliferation of ADSCs on CTi and MTi. MTi significantly supported the ADSC proliferation by day 4 compared to cells cultured on CTi; *p<0.05.](image)

3.4.3.6 Alkaline Phosphatase Activity

ALP is an early marker of osteoblastic differentiation. Differentiating osteoblasts are known to synthesize ALP enzyme during the initial stages of differentiation and it tends to decline as the osteoblasts enter into a maturation phase. Here, the differentiation ability of rabbit ADSCs on MTi and CTi were analyzed for a period of 21 days. The ADSCs on MTi showed a peak increase in ALP activity on day 7 and the activity reduced with increasing culture time (Figure 3.4.9). This early increase in ALP activity demonstrates the fact that
ADSCs on MTi entered into an early differentiation phase. As discussed with hMSCs, the presence of fibrin in the biopolymeric scaffolding is helping in the early differentiation and maturation of ADSCs compared to that of CTi. The presence of cell recognition sites such as RGD is reported to have a direct role in activating the differentiation pathways involved in osteogenesis and accelerating bone matrix production [248].

Figure 3.4.9: Alkaline phosphatase activity of ADSCs on MTi and CTi over a period of 21 days. Osteogenically induced ADSCs on MTi showed significant increase in enzyme activity on day 7 than control Ti. ** denoting p<0.01 for n=3 samples.

3.4.3.7 Biomineralization Studies

The biomineralization ability of MTi and CTi were carried out using osteogenically induced rabbit ADSCs at 3 different time points from 7 to 21 days. Here the SEM analysis showed the formation of calcium phosphates deposit on MTi by 14\textsuperscript{th} day onwards (Figure 3.4.10 A). By day 21, the surface of MTi plates were fully covered with calcium phosphate deposits. The deposits were further quantified using EDS which revealed a Ca/P ratio similar to bony hydroxyapatite which was not attained in the CTi (Figure 3.4.10 B). The EDS analysis of MTi plates at day 14 and 21 showed a Ca/P ratio of 1.6 and 1.76 which is similar to the ratio of bony hydroxyapatite reported in literature. Some calcium deposits were noticed on CTi plates, while no phosphorous presence was observed during the
analysis. Since the last stage of osteogenic differentiation is marked by the presence of calcium and phosphate in the mineralized nodules, the biomineralization studies indicated that MTi supported the complete differentiation of ADSCs and were behaving as osteoblast-like cells [297].

Figure 3.4.10: A) SEM images showing the biomineralized surfaces of each Ti plates after osteogenic induction and (B) EDS analysis of the mineralized Ti surfaces showing Ca/P ratio of 1.76 attained on MTi on day 21, similar to bone hydroxyapatite.

3.4.3.8 Biomineralized Bony Construct

For evaluating the efficacy of fibrin/alginate scaffolding approach in enhancing the osteointegration potential, implantation studies were carried out in New Zealand white rabbits. The animals were grouped into three: (i) CTi, (ii) MTi and (iii) MTi + cells. Along with CTi and MTi, osteogenically induced ADSC seeded MTi rods were also employed to analyze the potential of Ti together with autologous osteogenic induced stem cells in enhancing the osteointegration. ADSCs (isolated from three different rabbits of IIIrd group) seeded Ti rods after 21 days of culture was employed for implantation studies. As the implantation was to be done on three different rabbits, three groups of implants were maintained, with each group consisting of four Ti rods seeded with ADSCs isolated from three different rabbits. Of the four Ti rods seeded with autologous ADSCs, two of them...
were reserved for implantation onto the left and right tibia of animals. The additional two Ti rods seeded were taken for morphological studies to analyze the surface characteristics of implants before actual animal implantation. Complete coverage of the mineral deposits could be visualized from SEM morphological analysis of MTi rods (Figure 3.4.11 a). The mineral deposits were confirmed to be of calcium and phosphorous, with each showing a Ca/P ratio of ~1.8, similar to that of hydroxyapatite, the main inorganic mineral component of bone tissue (Figure 3.4.11 b).

In order to ensure the viability of the cell seeded construct during transportation from the cell culture facility to the surgical room, one set of implants seeded with osteogenically induced ADSCs were taken along with other Ti samples and assessed for the cell viability using Live/Dead assay after the implantation procedure. This was done to ensure that the ADSCs on Ti remained viable till the time of implantation. It was observed that the viability of the cells
on Ti rods was not affected, which was evident from the representative confocal microscopic image of ostegenically induced ADSCs on Ti rods (Figure 3.4.12).

![Confocal microscopic image of the live/dead staining showing the viability of ostegenically induced ADSCs on implanted Ti rods even after 4h outside the culture conditions. The viability was not affected upon transportation.](image)

**Figure 3.4.12**: Confocal microscopic image of the live/dead staining showing the viability of ostegenically induced ADSCs on implanted Ti rods even after 4h outside the culture conditions. The viability was not affected upon transportation.

### 3.4.3.9 Evaluation of Post Implanted Tissues- Gross Inspection

All rabbits tolerated the procedure well with no signs of inflammation or infection noticed at the implanted site. All the implanted Ti rods were stable and firmly fitting into the intramedullary cavity of the host bone tissue even after post implantation. No adverse foreign body reaction was observed on or around any implanted Ti rods.

### 3.4.3.10 Radiographic Analysis

X-ray micrographs were taken at regular intervals (immediately upon implantation ($D_0$), 1 month ($D_1$) and 3 months ($D_3$) post implantation) to check the position of implants in the bone tissue. The implants were stable and firmly fitting with no evidence of fracture or bone resorption. Both the lateral and antero-posterior aspects of tibias were recorded. No distinct radiographic differences were noted between CTi, MTi and MTi + cells as evident from the images depicted in Figure 3.4.13 and 14.
Figure 3.4.13: Representative X-ray images taken at regular intervals to confirm the position of implants. Both the right and left tibial images were captured. $D_0$-represents the radiographs taken immediately upon implantation and $D_1$-one month after implantation.

Figure 3.4.14: X-ray images taken at 3 months post implantation ($D_3$) after retrieving tibia. The high contrast images (circles on top right side) correspond to objects of known measurement kept to calibrate the images.
3.4.3.11 Interfacial Shear Strength Analysis

Push out test is normally done to analyze the interfacial strength developed over time between a biomaterial of interest and bone tissue. The interfacial shear strength analyzed by push out test revealed a significantly lower value for CTi implants compared to MTi and MTi + cells. The push out loads for CTi, MTi and MTi + cells after 12 weeks graphically represented in Figure 3.4.15 A were measured to be 57.82 ± 7.5, 128.9 ± 11.57 and 265.60 ± 65.25 N respectively. Their respective shear strength values were calculated to be 1.023 ± 0.133, 2.28 ± 0.20 and 4.69 ±1.15 MPa (Figure 3.4.15 B). Statistically significant enhancement in shear strength values was noticed on MTi and MTi + cells samples compared to CTi (##, ** p<0.01). Among MTi and MTi+ cells samples, the latter displayed a significantly high shear value (* p<0.05).

Figure 3.4.15: Represents the interfacial shear strength analysis data. (A) Graph representing the maximum force at which the failure take place and (B) The shear strength is calculated by dividing the maximum force with the surface area of the rods and is expressed as shear strength attributed by the different material. * denotes statistical significance compared between MTi and MTi+ cells samples (p<0.05), ## statistical significance compared between MTi and CTi (p<0.01) and ** represents statistical significance compared between MTi+ cells and CTi (p<0.01)

The increased shear strength value is often associated with the quality of bone at the implant interface. Some investigators have demonstrated a strong correlation between the apposed bone and shear strength. Friedman et al [298] have reported a correlation between shear strength and apposed bone area on a
rabbit intracancellous model whereas, no significant correlation was found in a study reported by Gotfredsen et al between the torque removal and bone contact area in their rabbit study [299]. SEM analysis and EDAX measurement done on the pushed out implants revealed a significant difference on the bone apposition layers on the tests and control implants (Figure 3.4.16). We could observe an extra bony apposition on MTi and MTi + cells implants compared to CTi. The presence of mineralized tissue was further confirmed by EDAX analysis on MTi and MTi + cells; while more unmineralized tissue was observed on CTi.

![Figure 3.4.16: SEM and EDS analysis of the surface of pushed out Ti rods. I a, II a and III a represents the CTi, MTi and MTi+ cells rods, b of all images represent a higher magnification SEM image and c represents the EDS data of each Ti surfaces. The attached bone on the surfaces was found to be higher on MTi and MTi + cells. The EDS analysis showed a Ca/P ratio of 1.8 for MTi and MTi + cells, whereas the CTi rods showed only little mineral deposition.](image)

The biopolymeric modification strategy has aided in enhanced bony apposition around the Ti rods. This was evident from the SEM and EDAX analyses of the pushed out implants. The interfacial shear strength was higher for the MTi + cells implants, which might be due to the presence of a preformed bony
layer on the surface. Here, the bone-bone interlocking might be occurring in place of the bone-implant interaction, which occurs with all metallic implants. The presence of an already formed bony layer can act as nucleation centre for the osteoblasts to adhere and deposit minerals. Similar results were reported in literature for a study involving osseointegration of surface-blasted implants made of titanium alloy and cobalt–chromium alloy in a rabbit intramedullary model [300]. Shear strength value of 2.6 ± 0.2 MPa was obtained for Ti6Al4V implants after 12 weeks of implantation in a rabbit intramedullary model. Surface roughness is yet another important factor reported in literature responsible for enhanced bone bonding. It is reported that a surface roughness value of ~ 1-2 µm is found to promote better osteointegration [301]. In the present work, the surface roughness monitored by AFM after surface scaffolding using fibrin-alginate showed an enhancement in $R_z$ values compared to bare Ti. For the MTi + cells case, though we have not analysed the surface roughness, the formation of a mineralized tissue could further enhance the surface roughness. This increase in surface roughness also might have contributed to the increased shear strength values demonstrated by surface modified implants.

3.4.3.12 Histology and Histomorphometric Analysis

Qualitative non-decalcified histology analysis of the different implanted Ti samples was analyzed by Stevenel’s blue and Van Gieson's picrofuchsin after 12 weeks of implantation. Stevenel's blue stains cells and fibrous tissue in blue tones and Van Gieson's picrofuchsin colours bone in orange and osteoid matrix in yellow green. No necrosis or inflammatory responses were observed on or around any implants as evident from Figure 3.4.17. In all the histologic sections, the Ti cylindrical rods are represented in black, old bone in yellow, new bone in orange, fibrous tissue in blue and osteoblasts in black. Good bone apposition with no gaps was noticed on the MTi and MTi + cells implants. Rabbits implanted with CTi rods were found to have a thick fibrous tissue encapsulation around the implants as depicted in Figure 3.4.17 (ai-aiii). Intervening fibrous tissue at the bone-implant interface is associated with lack of osteointegration. On the contrary, a thin layer of mature lamellar bone could be seen around the MTi implants (Figure 3.4.17...
(bi-biii) whereas, a thick layer of mature lamellar bone was observed around MTi + cells (Figure 3.4.17 (ci-ciii)). On the MTi and MTi + cells, a good haversian system was formed around the bone implant contact area, signifying good remodelling process occurring at the implant interface. The newly formed bone layer was more or less continuous and directly in contact with the implant surface.

The new bone formation around the MTi + cells was more extensive than that of MTi implants. One probable reason for the improved bone apposition around the MTi + cells (IIIrd) implants could be the presence of a pre-formed biomineralized apatite layer on the surface. An essential requirement reported in
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literature for a material to bond with the living bone tissue is the formation of a biologically active bone-like apatite layer on material surface [302]. The presence of such a layer on the implant surface could aid in enhanced bonding and neo-osteogenesis [303]. Bone formation around Ti implants depends mainly on the surface treatments done on the implant surface. Here, our strategy of providing a coating of fibrin/alginate and its subsequent cellular treatment on Ti has resulted in a substrate on which bone can be directly formed. The osteoconductive property of fibrin is well established in many literatures [304, 305]. It has binding pockets for osteoprogenitors and fosters them to differentiate into mature osteoblasts. Enhanced bone formation was reported in a study when platelet rich fibrin (PRF) was used along with Tricalcium phosphate (TCP) to graft the maxillary anterior sinus walls of New Zealand white rabbits. The PRF-TCP demonstrated more rapid bone healing than the recombinant human bone morphogenic protein 2 (rhBMP-2) - coated TCP or the TCP-only control [306]. In another study reported by Perka et al a positive effect on bone formation was observed when fibrin sealant along with periosteal cells were implanted in bone defects in rabbits [307].

Fibrin has also been reported to have an osteoinductive nature; however it has been a subject of controversy. In a study by Albrektsson et al [308] on rabbit models using dividable titanium implants treated with and without Fibrin Adhesive System (FAS), there was less bone formation observed in implants treated with fibrin sealant at 5 weeks than in the implants alone. Isogai et al [309] in his studies also could only observe newly formed bone structures in all cell/matrix-fibrin glue admixtures, but none in fibrin glue injected alone sites. On the contrary, Abiraman et al [131] had demonstrated the osteoinductive properties of fibrin sealant in mice models. Neobone formation was observed at 28 days on hydroxyapatite and bioglass coated with fibrin sealant implanted heterotropic sites in mice. Likewise, Yamada et al also demonstrated the osteoinductive property of fibrin sealant in combination with beta-tricalcium phosphate and mesenchymal stem cells when they were implanted in the heterotropic sites in rat [310]. Our detailed analysis using fibrin-alginate scaffolded Ti rods in rabbit intramedullary
model demonstrated improved bone formation in comparison to control Ti rods without the scaffolding.

The histomorphometric results were consistent with the mechanical data. As with the interfacial strength analysis, the bone-implant contact percentage was in the order MTi + cells > MTi > CTi. The percentage bone contact around MTi + cells was found to be 82.57 ± 2.77 for MTi implants it was 55.57 ± 2.13 and for CTi, it was around 26.28 ± 5.19 (Figure 3.4.18). A positive correlation could be noticed between the histopathological analysis and shear strength analysis at the bone-implant interface. Noticeable enhancement in bone contact was observed on MTi + cells and MTi implants compared to CTi. Statistically significant enhancement in bone contact percentage was noticed on MTi and MTi + cells samples compared to CTi (* represents comparison between MTi and CTi with p value <0.05 and ## represents statistical difference between MTi + cells and CTi with p<0.01). Among MTi and MTi + cells samples, the latter displayed a significantly higher percentage of bone contact (** p<0.01). Due to the bioinert nature, commercially pure Ti does not have the high mechanical ability to bond to bone [311]. Our results also demonstrated the similar trend of lesser bone contact around CTi compared to the surface modified implants.

Figure 3.4.18: Representative relative quantification of the percentage bone contact around MTi, MTi+ cells and CTi are shown. * represents comparison between MTi and CTi with p value <0.05, ## represents statistical difference between MTi+ cells and CTi with p<0.01 and ** represents significance between MTi and MTi + cells with p<0.01.
In our study, we have utilized rabbit intramedullary model for studying the osteointegration potential of the surface scaffolding approach. Several other implantation models such as transcortical [312], transmetaphyseal [313] and intracancellous [314] have also been proposed. But due to the clinical relevance associated with intramedullary implantation, especially utilized for total hip arthroplasty, we preferred to exploit the intramedullary implantation model. A short implantation time of 12 weeks was carried out in our investigation as the average time required for bone integration reported in literature is 3 months [315]. Within this short span we could observe mature lamellar bone formation around the MTi and MTi + cells, with more extensive distribution around the latter. The effect of fibrin/alginate together with the osteogenic induced ADSCs proved to be a better construct, as it minimized the delay in osteogenesis caused by the migration and expansion of native osteoprogenitors.

One of the important aspects to be considered when implantation of biomaterials is the immune response occurring in the host tissue [276]. Implantation of foreign materials often results in injury, followed by a cascade of cellular events. It is these cellular events that affect the extent or degree of granulation tissue formation, foreign body reaction, and fibrosis or fibrous capsule development [316]. Prolonged inflammatory reactions at the injury site can lead to impaired wound healing, resulting in impaired tissue regeneration. The surface topology, size, shape and physical properties govern the time duration of the inflammatory and wound healing processes. Hence, any biomaterial before implantation should satisfy certain requirements, as set by ISO, ASTM, and USP etc [317]. Osteointegration or bone bonding is often determined by the absence of necrosis and inflammation and by the presence of newly formed bone at the host bone-material interface. We could not find any intervening space at the bone-implant interface of MTi and MTi + cells. SEM analysis of PMMA embedded bone-implant transverse sections clearly showed the presence of an intervening soft tissue at the bone-implant interface of CTi implants, whereas no such gaps could be noticed for the surface modified implants (Figure 3.4.19). Hence the presence of new deposited bone with minimal fibrous tissue encapsulation at 12 weeks post implantation around surface scaffolded Ti indicated the potential of
the coating process in inducing acceptable *in vivo* response. Our results also support numerous earlier reports that biomimetic coating of Ti with ECM components can stimulate the bone healing around metallic implants [318, 319].

Figure 3.4.19: SEM images from cross sections through the implants and bone tissue showing the interface zone at 12 weeks post implantation. Intervening gap is noticed on (a) CTi rods whereas, no such gaps were observed on (b) MTi and (c) MTi + cells implants.

However, for the clinical translation of this surface scaffolding approach further long term implantation studies should be carried out in higher animal models. The performance of the modified Ti under loading and challenging scenarios such as immediate loading should be evaluated prior to translating this approach.

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